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Exhibit A

DETECTION OF MAREK'S DISEASE VIRUS ANTIGENS AND DNA IN FEATHERS FROM INFECTED CHICKENS

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Two novel tests, enzyme-linked immunosorbent assay (ELISA) and dot-blot hybridization, were developed to detect and quantify the antigens and DNA of Marek's disease virus (MDV) in feather tips from infected chickens. In both methods, buffered extracts of the feathers served as the same test material. The ELISA technique was compared to the conventional agar-gel precipitation (AGP) test, using the same convalescent serum from a MDV-infected bird. Of 86 feather samples tested, 34 were negative by both methods, while 6 out of 52 were ELISA positive but AGP negative. Viral antigen detection by the AGP and ELISA methods was compared with the detection of MDV DNA by the dot-blot DNA hybridization technique. At an ELISA reading (OD 405) of 0.3 and above, only 5 out of 48 DNA extracts failed to hybridize with the MDV-DNA probe. The use of the radioactively labelled MDV-DNA probe for hybridization with DNA extracts from feather tips of MDV-infected chickens was both sensitive and specific, and there was good correlation among the different tests.

Marek's disease virus feather tips MDV-specific antigens enzyme-linked immunosorbent assay (ELISA) MDV DNA MDV-DNA diagnostic hybridization

INTRODUCTION

Marek's disease virus (MDV) causes a disease of chickens characterized by production of lymphomas. The virus spreads horizontally and is responsible for major economic losses in affected flocks. MDV is present in a cell-associated form in most organs and tissues of infected chickens (Phillips and Biggs, 1972).

Marek's disease is diagnosed on the basis of flock history, age of the bird, and typical clinical signs. The principal symptoms are paralysis, enlargement of, and malignant tumor transformation in, the viscera, skin and other organs. Histologically, there is infiltration of a mixed lymphocyte population. These data, as well as virus isolation and antibody demonstration, are considered pathognomic. Virus isolation is performed by inoculating blood, buffy coat, or single cell suspensions of organs from suspected chickens onto preformed monolayers of chicken kidney (CK) or duck embryo fibroblast (DEF) cells (Witter et al., 1969). MDV is highly cell-associated, so it

takes 7–10 days before the cytopathic effect becomes apparent. Positive cultures are scored by immunofluorescent (IF) staining of the typical foci which are composed of spherical cells. Presence of the virus in infected chickens can also be visualized by the agar precipitation test (AGP), where feather tips are reacted with convalescent sera (Haider et al., 1970; Marquardt, 1972; Rangga-Tabbu and Cho, 1982; Zanella et al., 1984). This method is simple and rapid to perform, but relatively insensitive. Elevated specific serum antibodies can also serve for monitoring MDV infections in diagnostic tests that include AGP, virus neutralization using cell-free virus prepared from the skin and feathers, and IF (Sharma, 1975). These tests are very specific but reveal viral infections only at more remote intervals from initiation of the infection, while their sensitivity depends on the age, the immunological status of the birds and the virulence and antigenicity of the infectious virus.

A productive MDV infection, resulting in the release of cell-free infectious virus, is consistently present in the feather follicle (Calnek and Hitchner, 1969; Calnek et al., 1970a,b,c). After maturation in the superficial layers of the feather follicle epithelium virus particles are released into the space between the follicular epithelium and the rachis (Nazarian, 1971). Based on the study of Davis and Sharma (1973), the production of cell-free virus in the feather follicle epithelium was explained by the psychological process of keratinization, because lysosomal activity is at a reduced level in these cells. Appreciable amounts of virus accumulate in the feather follicle space and the feather tip cavity. The feather tips are, therefore, the organ of choice for demonstrating and monitoring MDV infection of chickens and enable measurement of virus-specific components, namely antigens and DNA. Recent advances in the area of recombinant DNA technology have led to the use of virus DNA cloned in bacterial plasmids as nucleic acid probes in the diagnosis of viral infections. Virus sequences present in different organs may be assayed and quantified. This technique allows the study of the epidemiology of virus infections at the molecular level (Bransma and Miller, 1980; Kulski and Norval, 1985).

We describe an enzyme-linked immunosorbent assay (ELISA) method which was developed to measure MDV-specific antigens in feather tips. The results obtained by this technique were verified by AGP. In addition, both a qualitative and quantitative method for detecting MDV DNA was used, and its sensitivity was compared with that of detecting MDV antigens in feathers.

MATERIALS AND METHODS

Viruses and chickens

Leghorn SPF embryonated eggs obtained from SPAFAS (CT, USA) were hatched and immediately transferred to isolator units. The chickens were divided into 5 groups: 4 groups were inoculated at 7 days of age with the GA_5 strain [MDV serotype 1 (Calnek, 1973)] and with 3 Israeli field isolates of MDV serotype 1: A, G and M. The fifth group served as uninfected controls.

Chicken serum

Control serum was taken from SPF chickens aged 60–90 days. Convalescent serum came from GA_5-infected chickens that were bled after 90 days of infection.

AGP test

The reaction was performed in Petri dishes (5 cm diameter) which contained 5 ml of 1% Noble agar in 8% NaCl buffered at pH 7.4 with phosphate-buffered saline (PBS). A rosette pattern of six holes containing the feather tips surrounded the hole with the convalescent GA_5 serum. Wing feathers were collected from the infected and control groups of chickens (26–60 days postinfection) and kept in plastic bags at –90°C until tested. Portions of about 0.5 cm of the feather tip were cut and put into 0.3 mm diameter holes for the AGP reaction. In each hole we put one tip from a large feather, but more than one tip was used when the feathers were smaller. The results were read after 24–48 h incubation of the plates at 37°C in a humid chamber.

Extraction of viral antigens from feather tips and feathers

Feather tips were cut into 0.5 cm pieces and incubated in buffer at 4°C for 24–48 h to extract the viral antigen. Each extract was prepared from ten pieces of tips of different feathers in 1 ml of buffer. For the ELISA test, the extraction was performed in either phosphate-buffered saline (PBS) or in 0.05 M carbonate–bicarbonate buffer, pH 9.6. After extraction, the buffer and tips were kept at –20°C until tested.

Extraction of virus nucleic acids from feather tips

Virus nucleic acids were extracted by a modification of the method described by Shlomai et al. (1976). Ten feather tips, each 0.5 cm in length, were incubated overnight at room temperature in 1 ml TE buffer (1 mM EDTA and 10 mM Tris–HCl, pH 8.0). Virions were released from the tips by sonication (3 times for 15 sec) in a waterbath sonicator. Nucleic acids were prepared by incubation of the sonicated extract either overnight at room temperature or for 2 h at 37°C with of 1% sodium dodecyl sulfate (SDS) and 1 mg/ml proteinase K (Sigma Chemical Co., St. Louis, MO). An equal volume of phenol was mixed with the SDS–proteinase K-treated material. The aqueous phase was extracted twice with two volumes of chloroform:isoamyl alcohol (24:1). The nucleic acids were precipitated with ethanol for 30 min at –80°C (or overnight at –20°C), centrifuged, dried, and resuspended in 50 µl TE buffer.

ELISA test

Individual wells in polystyrene plates (Nunc, Roskilde, Denmark) were first coated with buffered extracts of the feather tips by incubation of the plates overnight at 4°C. All steps of the ELISA reaction were performed in volumes of 0.1 ml per well. After the plates had been rinsed 3 times with PBS-Tween 20 (0.05%), either serum from uninfected SPF chickens or convalescent GA_5 serum diluted in PBS-BSA (0.02%), was added to the antigen-coated wells. After incubation at 37°C for 1 h, the plates were

rinsed again, an immunoglobulin conjugate was added, and the plates incubated for a further 2–3 h at 37°C. The rabbit anti-chicken IgG was conjugated either to alkaline phosphatase (AP) or to horseradish peroxidase (HPRO) (Bio-Yeda, Rehovot, Israel). The plates were washed again, and the respective substrate solutions were added to the wells. For AP, the substrate was 1 mg/ml of *p*-nitrophenyl phosphate (Sigma) in 10% diethanolamine buffer (pH 9.8); HPRO was reacted with 1 mg/ml of azino di-3-ethyl-benzthiazoline sulphonate (ABTS) (Sigma), in 0.1 M citrate–0.2 M phosphate buffer, pH 4.0, supplemented with 0.003% H_2O_2. The absorbencies were read in an automatic MR 580 Dynatech ELISA reader at A_{405} nm. AP was routinely used for estimating antigen content of feathers.

Dot-blot hybridization of DNA-nucleic acid extracts

The dot-blot hybridization procedure was performed according to the instructions supplied with the Minifold (Schleicher and Schüll, Dassel, W. Germany). The concentration of the nucleic acid in the extracts from feather tips was determined by measuring the optical density at 260 nm in a Gilford spectrophotometer 2400-S (Oberlin, OH, USA).

Various dilutions of the nucleic acid extracts were prepared in TE8 buffer in a final volume of 20 µl. 1.5 µl of 1 mg/ml salmon sperm DNA was used as a carrier and 300 µl of denaturation solution (100 mM Tris–HCl, pH 7.4, 2 N NaOH and 20 × SSC (Maniatis et al., 1982) in a volume ratio of 17 : 3 : 10) were added. The solution was incubated for 10 min at 80°C to denature the nucleic acids. The pH was adjusted to between 7 and 8 by adding 50–70 µl of 2 M Tris–HCl. The nitrocellulose membrane was soaked with water and then with 2 × SSC and placed on the Minifold. The denatured nucleic acid preparations were added into the wells and sucked gently through the nitrocellulose membrane. The membrane was dried and baked at 80°C under vacuum for 1–2 h.

The EcoRI-N fragment (2.8 kb) of MDV DNA cloned in pBR328 (obtained from Dr. R. Silva, Regional Poultry Research Laboratory, East Lansing, and originally prepared by Gibbs et al., 1984) was radioactively labelled by using a nick-translation reagent kit (Bethesda Research Laboratories) (Maniatis et al., 1982) and was used as the probe for DNA hybridization. Prehybridization and hybridization procedures were performed at 50°C in 5 × SSC salt conditions and autoradiography was done at –70°C as described by Maniatis et al. (1982).

Five dilutions from each extract were spotted in a vertical lane on the nitrocellulose membrane using the Minifold. The probe dilution lane was localized one or two lanes away from the extract lanes to avoid contact between spots after autoradiography. The density (degree of darkening) of the spots in the developed X-ray films was measured in a densitometer (Helena Laboratories, Model No. 1053, TX, USA).

The correlation between the density of each spot in the developed X-ray films and the amount of hybridized nucleic acid on the filter was calculated assuming self-probe hybridization as a 100% value. The density of the self-probe hybridization spots

represents the known amount of probe DNA on the filter. The spots in the lanes of the feather tip extracts result from hybridization with the MDV-N fragment (2.8 kb). Each MDV DNA molecule (175 kb) contains one N fragment. The amount of N fragment in each extract was determined by comparing the density of each spot with that of the self-hybridization spot. Depending on the amount of MDV-N fragment, the total amount of MDV DNA and its percentage in each extract were calculated.

RESULTS

Determination of the ELISA parameters for the detection of MDV antigens

The optimal ELISA parameters for the detection of MDV antigens were determined. Feather tips were extracted in each of two buffers (0.05 M carbonate-bicarbonate buffer, pH 9.6 and PBS), and 0.1 ml served for the coating of each well. Two sera were reacted in parallel: anti-GA_5 and SPF at a 1 : 200 dilution. The reaction was performed with two separate enzymes, horseradish peroxidase and alkaline phosphatase, with the appropriate substrates.

Table 1 shows the results of a typical ELISA test as performed in duplicate wells. The anti-GA_5 serum reacted strongly with feather tip extracts obtained from MDV-infected chickens but did not react with extracts of feather tips from uninfected SPF

TABLE 1

Determination of the ELISA parameters for MDV antigen detection in extracts of feather tips

Serum	Horseradish peroxidase system[a]				Alkaline phosphatase system[b]				MDV DNA detection[c]
	Ag coating in PBS		Ag coating in carbonate buffer		Ag coating in PBS		Ag coating in carbonate buffer		
Serum	MDV	SPF	MDV	SPF	MDV	SPF	MDV	SPF	
Chicken									
Uninfected:									
(1)	0.0	0.0	0.0	0.0	0.01	0.01	0.01	0.0	Negative
(2)	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	Negative
Infected GA_5 virus:									
(1)	0.19	0.0	0.27	0.0	0.19	0.0	0.29	0.04	Positive
(2)	0.59	0.0	0.76	0.0	0.45	0.0	0.47	0.02	Positive
(3)	0.91	0.0	0.93	0.0	0.61	0.0	0.56	0.06	Positive
Infected M isolate:									
(1)	0.71	0.0	1.09	0.0	0.52	0.0	0.57	0.01	Not done

[a] ELISA values read on 405/490 nm.

[b] ELISA values read on 405/630 nm.

[c] MDV DNA was determined by dot-blot hybridization test, using PBS extraction of feather tips (see Fig. 4).

chickens. Serum from SPF chickens gave negative reactions when used with antigens extracted from infected feathers. These results were seen in all ELISA test combinations.

The presence of MDV DNA was determined by the dot-blot hybridization method on the same feather tip extracts (Table 1). MDV DNA was detected only in extracts of MDV-infected chickens.

Optimal antibody dilution for MDV antigen detection in ELISA

Determination of the optimal antibody dilution in the ELISA test was made with convalescent pooled chicken serum (AGP positive). The serum (in 2-fold dilutions) was reacted with extract prepared from AGP-positive feather tips from a GA_5-infected chicken. Serum from SPF chickens was used as control. The results presented in Fig. 1 revealed antibody dilutions ranging from 1 : 50 to 1 : 800 that had a strong specific reaction. The reaction with SPF serum was low and was subtracted from the absorption value obtained with anti-GA_5 serum (Fig. 1). The 1 : 200 dilution of the MDV(GA_5) convalescent serum was therefore chosen for routine estimation of antigen content of feathers.

Localization of MDV antigen along the feathers

To define the exact localization of MDV antigens on the feathers removed from infected chickens, we checked two representative kinds of wing feathers: (a) soft and blood-filled feathers and (b) dry and empty wing feathers (Fig. 2A and B, respectively).

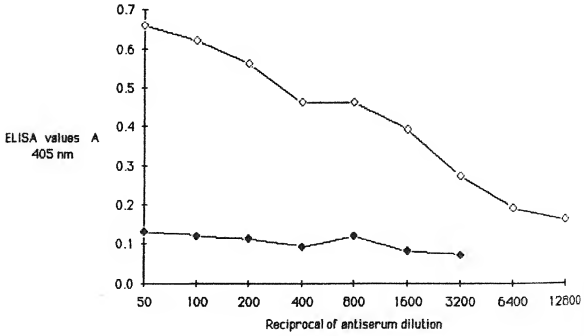

Fig. 1. Determination of the optimal antibody dilution for the detection of MDV antigens in feather tip extracts in the ELISA test. ◇——◇, GA_5 serum reaction; ◆——◆, SPF serum reaction.

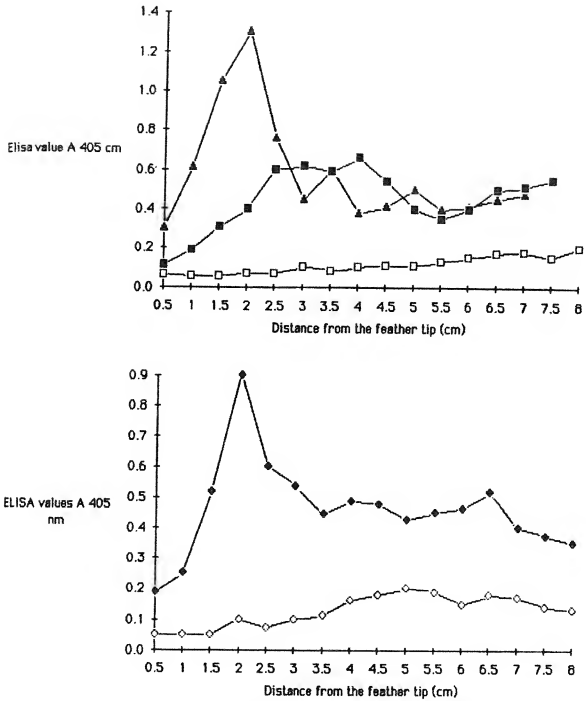

Fig. 2. Distribution of MDV antigen on feathers of GA_5-infected birds. Blood-filled feathers (A) and dry and empty feathers (B) were tested in the ELISA reaction against anti-GA_5 serum (full symbols) and SPF serum (open symbols). Feathers were taken from a chicken 26 days p.i. (▲) and from a chicken 60 days p.i. (□, ◇).

Such feathers were cut into 0.5 cm sections over their entire length. Adjacent sections from each feather were extracted in 0.1 ml buffer. The extracted antigens were reacted in the ELISA test with both anti-GA_5 and SPF sera. The distribution of MDV antigens along several feathers tested in each group is shown in Fig. 2A and B. The largest amounts of MDV antigen were found 2.5–3.0 cm from the point of insertion of the feather, but viral antigen was present along the entire length of the feather. Since this pattern was common to both types of wing feathers, both may serve as a source of MDV antigen. Feathers taken from uninfected chickens did not react with either antiserum (data not shown).

Correlation between AGP and ELISA tests for detection of MDV antigens

Feathers collected from uninfected and MDV-infected SPF chickens were tested in both the AGP and ELISA tests. The AGP test was scored individually for each chicken and the ELISA reading plotted as the net optical density (OD) at $A_{405\,nm}$ (the optical density of the anti-GA_5 serum minus that of the SPF serum reacted on the same extract). The percentage of AGP-positive tests was calculated and plotted at 0.1 ($A_{405\,nm}$) intervals (Fig. 3). Of 86 feather samples tested, 34 were negative by both tests. At an ELISA OD_{405} of 0.2, only 1 out of 5 samples was AGP positive, and of the remaining 47 samples with an OD_{405} of 0.3 or more, 2 were AGP negative. In all, 6 out of 52 samples were ELISA positive and AGP negative. Above the $A_{405\,nm}$ value of 0.6, all of the chickens had positive AGP reactions. There was thus very good correlation between the ELISA and AGP results, but the ELISA was more sensitive.

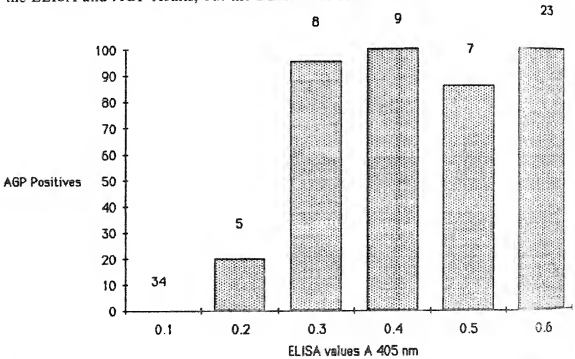

Fig. 3. Correlation between the AGP and the ELISA tests for detecting MDV antigens. Brackets indicate number of samples at each point.

Specificity and sensitivity of MDV DNA dot-blot hybridization technique

The dot-blot hybridization technique was used to detect the presence of MDV DNA in the extracts from feather tips. The technique was to titrate DNA extracts taken from feather tips of three GA_5-infected chickens, two SPF chickens, and from a chick-embryo fibroblast (CEF) cell culture. Five dilutions (10^{-1} to 10^{-5}) were prepared from the original extracts and spotted onto nitrocellulose paper. The spots were reacted with the MDV probe, radiolabeled with ^{32}P by nick-translation (500,000 cpm/1 ng probe DNA). Fig. 4 (lanes 1 and 2) shows the self-hybridization of the probe, the basic parameter for calculating the amount of MDV DNA in the tested specimens. Specific reactions were observed up to the 10^{-3} dilution of DNA extracted from the feathers. The CEF reaction represented the background hybridization of the chicken cellular DNA (Fig. 4, lane 3). Only background hybridization was observed with the MDV DNA probe. Extracts of feather tips from GA_5-infected chickens (Fig. 4, lanes 4, 5 and 6) showed hybridization of the MDV DNA probe only in the first dilution, implying an approximate 1 ng sensitivity. Uninfected chickens did not show any hybridization with the probe (Fig. 4, lanes 7 and 8). The EcoRI-N fragment of MDV DNA used as a probe did not hybridize to either type 3 (HVT) or type 2 (SB1) DNA preparations from virions and infected fibroblasts (not shown).

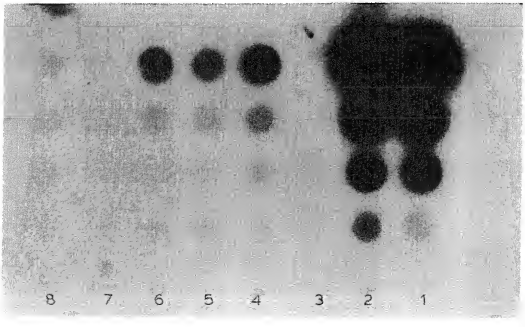

Fig. 4. Specificity and sensitivity of MDV DNA dot-blot hybridization technique. Lanes 1 and 2: self-hybridization of the MDV DNA probe; lane 3: DNA extracted from cultured chick-embryo fibroblasts; lanes 4, 5 and 6: feather tip DNA extracts of GA_5-infected chickens; lanes 7 and 8: feather tip DNA extracts of uninfected chickens.

Correlation between MDV-antigen detection methods and the MDV-DNA detection method

Two methods were employed to demonstrate the presence of MDV-specific antigens in feather tips: the AGP and the ELISA method. It was of interest to compare the results obtained with these techniques with the detection of MDV DNA in the extracts from the same feather tips, as measured by the dot-blot hybridization technique. For this purpose, 65 extracts of feather tips were studied by the three methods. Table 2 summarizes the results of MDV detection in the feather tips of four groups of MDV-infected chickens and one group of SPF chickens. Uninfected SPF chickens gave negative results by both methods. In the GA_5-infected birds, the great majority (6/8: 75%) were positive for DNA (db^+) and for antigens (AGP^+), only 1 out of 8 chickens (12.5%) was positive for viral antigens and negative in the dot-blot test for DNA (db^-) and 1 chicken (12.5%) was negative for viral antigens (AGP^-) but positive for viral DNA. Among the birds infected with the MDV field isolate A, there were 15/18 (83%) positive chickens in AGP and dot-blot DNA tests, while 3 chickens (16.7%) were negative in both tests. In the groups of chickens infected with the MDV field isolate M, only 10/17 (58.8%) chickens were positive by both tests, while 4 chickens (23.5%) were negative by the two methods. Of the remaining chickens, 2 (11.7%) were positive for viral DNA, and 1 (5.8%) was positive for viral antigen. Of the chickens infected with the MDV field isolate G, only 8/22 (36.3%) were positive by the two methods, while 12/22 (54.5%) were negative by the two methods. One chicken was positive in the AGP test only, and another was positive in the DNA test. Thus Table 2 shows that 60% of the feather tips of MDV-infected chickens were positive by the tests for MDV DNA or MDV antigens. Another 11% of the chickens could be detected by either one of the tests, while 29% of the chickens were negative by both tests. This represents a total concordance of 90.3%.

The positive or negative dot-blot hybridization findings were further correlated with

TABLE 2

Correlation between the AGP method for detecting MDV antigens and the dot-blot (db) method for detecting MDV DNA

MDV isolate	No samples	AGP^+ db^+	AGP^+ db^-	AGP^- db^+	AGP^- db^-
GA_5	8	6	1	1	0
A	18	15	0	0	3
M	17	10	1	2	4
G	22	8	1	1	12
Total	65 (100%)	39 (60.1%)	3 (4.6%)	4 (6.1%)	19 (29.2%)
SPF	14	0	0		0

the ELISA absorbencies in Fig. 5. There appears to be a quantitative relationship between the ELISA optical density which is indicative of the amount of viral antigens and the percentage of MDV DNA positive specimens. The results were plotted after calculating the MDV DNA percent of positives at defined ranges of 0.1 A_{405}. From a value of 0.3 A_{405}, half of the feather samples contained virus DNA and above 0.5 A_{405}, all the specimens were positive. At an ELISA OD_{405} of 0.3 and above, only 5 out of 48 DNA extracts failed to hybridize with the MDV DNA probe.

Quantitative comparison between ELISA values and the relative amount of viral DNA in feather tips

The ability to measure two different viral components on the same feather specimen was analyzed quantitatively. Based on the intensity of radiolabelled spots on the X-ray films, we calculated the percentage of viral DNA in the sample as related to the total DNA found in the feather tip extract. A scatter plot was prepared by plotting the percentage of MDV DNA in the extracts versus the ELISA OD at A_{405} obtained with the same feather tips. As can be seen in Fig. 6, the values for the different feather extracts were widely distributed, but a general trend was evident. Increasing viral DNA contents in the feather tip extracts correlated with higher ELISA absorbencies. Moreover, a linear regression analysis was calculated for the 77 specimens tested. The correlation coefficient (r) calculated for this regression line is 0.555, which is statistically significant at a $P<0.001$ level.

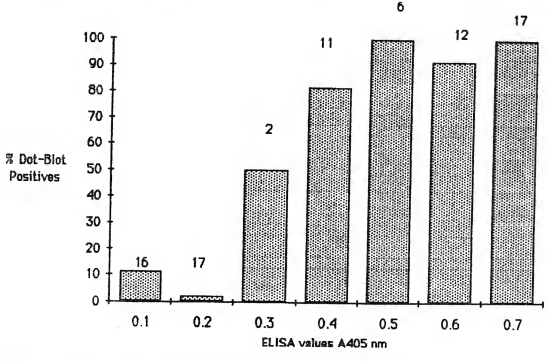

Fig. 5. Correlation between the ELISA method for detecting MDV antigens and the dot-blot hybridization method for detecting MDV DNA scored on a positive-negative level. Brackets indicate number of samples at each point.

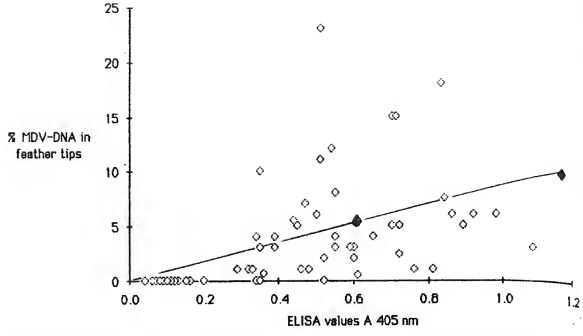

Fig. 6. Quantitative relationship between ELISA values for MDV antigens and the relative amount of viral DNA in feather tips.

DISCUSSION

The ELISA and dot-blot DNA hybridization methods for demonstrating MDV proteins and DNA are easily adapted for diagnosis of chickens suffering from Marek's disease. Both methods are rapid, highly reliable, and relatively simple to perform. They have obvious advantages over the standard approach of demonstrating infectious particles in cell cultures inoculated with blood or organs from affected chickens (Witter et al., 1969). Virus isolation requires a relatively long period before live virus can be detected by IF or histochemically, using biotinylated lectins (Malkinson et al., 1986), while both ELISA and DNA dot-blots can be completed in 24 h.

The ELISA test for measuring MDV antigens in feather tips is considerably more sensitive than the AGP test published 15 years ago (Haider et al., 1970). The AGP method allows only qualitative demonstration of precipitation lines, whereas quantitative measurements of the antigen content can be performed by ELISA. Although we are presently unable to quantify or characterize individual virus-specific antigens in the feather tips by this method, it has been possible to measure the total viral antigen content of feathers from chickens infected with various strains of the virus. On the other hand, viral DNA can be assayed precisely, based on the density of the MDV DNA hybridization dots.

The reliability of the DNA and antigen-measuring methods was assessed by comparing the results of the tests done by these two methods on the same specimens. The AGP and the dot-blot tests recorded as either positive or negative revealed viral

components in 60% of the specimens. In the ELISA, a quantitative relationship was found at absorption values over 0.5, when all the specimens were also positive for MDV DNA. There was a good correlation between the ELISA and the AGP tests, but the ELISA proved to be a more sensitive method. A comparison of the values measured for the MDV DNA with the ELISA values for the viral antigens, showed a statistically significant relationship (Fig. 6). An increase in the viral antigen content in the feather tips was accompanied by an increase in the viral DNA content (probably due to more viral particles in the feather tips). Furthermore, the point dispersion of Fig. 6 reveals different levels of viral content in the feather tips of chickens infected with the four MDV viruses. Within individual limits of sensitivity, the three diagnostic methods for the detection of viral antigens, and DNA therefore provides a reliable basis for the laboratory diagnosis of MDV.

Knowledge of the MDV antigens and DNA content in feather tips will provide additional information on the shedding patterns of different MDV serotype 1 isolates and their ability to infect contact chickens.

MDV is the only avian virus whose major route of excretion by the infected bird is by the feather epithelium. The ease with which feather samples can be taken makes them a most attractive feature in the repertoire of infective materials used for diagnostic purposes. We have shown that both the ELISA and DNA hybridization are more sensitive for detecting viral particles than AGP, and more rapid and easier to perform than virus neutralization. Because DNA probes are normally radioactively labelled, ELISA emerges as the routine diagnostic method of choice at present.

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Exhibit B

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Research Note—

Polymerase Chain Reaction for Detection of Avian Leukosis Virus Subgroup J in Feather Pulp

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SUMMARY. Feather pulp from experimentally infected chickens was used as a source of DNA for polymerase chain reaction (PCR) amplification of avian leukosis virus subgroup J (ALV-J) proviral DNA. A primer set that produces a large amplicon (~2125) was used to detect ALV-J proviral DNA. This primer set was used in lieu of previously published primers because it allows for sequencing of the entire envelope gene and because it was able to detect diagnostically a number of North American ALV-J isolates that could not be detected with previously published primers and PCR conditions. ALV-J proviral DNA was detected in feather pulp at 7 days of age in more than 90% of birds infected as embryos and 7 days postinoculation in over 50% of chickens infected at 3 days of age. The results obtained with PCR on feather pulp were compared with those of virus isolation. In the embryo-inoculated birds, the percentages of agreement between PCR and virus isolation were 92.5% at 7 days of age and 100% at 28, 42, 49, and 56 days of age. However, the overall sensitivity of virus isolation in embryo-infected birds was higher, particularly at 7 and 56 days of age. In chickens inoculated at 3 days of age, the percentages of agreement of detection between PCR and virus isolation ranged from 75% at 10 days of age to 100% at 42 days of age. Agreement of negative results of ALV-J detection by PCR and virus isolation in chickens infected post-hatch ranged between 66.6% and 100% between the ages of 10 and 42 days. Virus isolation requires chicken embryo fibroblasts of specific genetic lines, and the process takes on average 7–9 days. Aseptic collection of blood and tissues for virus isolation and molecular detection of ALV-J requires sterile necropsy instruments as well as syringes and needles for each individual chicken, whereas sterile microcentrifuge tubes and gloves are the only equipment necessary for aseptic feather pulp collection for ALV-J detection by PCR. PCR-based detection of ALV-J in feather pulp is especially suitable when ALV-J infection must be diagnosed rapidly and unequivocally without killing the chicken(s) and in situations where crucial reagents or suitable virus propagation substrates are not readily available for isolation and propagation of ALV-J in cell culture.

RESUMEN. *Nota de Investigación*—Detección del virus de la leucosis aviar subgrupo J a partir de la pulpa de la pluma mediante la prueba de reacción en cadena por la polimerasa.

Se empleó la pulpa de las plumas de aves infectadas experimentalmente como fuente de ADN para la amplificación del provirus ADN del virus de la leucosis aviar subgrupo J mediante la prueba de reacción en cadena por la polimerasa (PCR). Para detectar el provirus ADN del virus del subgrupo J de leucosis aviar se usaron iniciadores que amplifican una porción de 2,125 pares de bases. Se emplearon dichos iniciadores en lugar de iniciadores publicados anteriormente debido a que permiten secuenciar completamente el gen de la envoltura del virus y por que pueden detectar un número de aislamientos del virus J de leucosis aviar de América del Norte que no pudieron ser detectados con los iniciadores publicados anteriormente. Se detectó el provirus ADN del virus J en la pulpa de la pluma a los 7 días de edad en más del 90% de las aves infectadas a edad embrionaria y a los 7 días posteriores a la inoculación en más del 50% de las aves infectadas a los 3 días de edad. Se

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compararon los resultados obtenidos mediante la prueba de PCR a partir de la pulpa de las plumas y el aislamiento del virus. Se observó una concordancia del 92.5% entre la prueba de PCR y el aislamiento del virus a los 7 días de edad y del 100% a los 28, 42, 49 y 56 días de edad. Sin embargo, la sensibilidad del aislamiento del virus en aves infectadas a edad embrionaria fue mayor, especialmente a los 7 y 56 días de edad. En las aves inoculadas a los 3 días de edad, los porcentajes de correlación entre la prueba de PCR y el aislamiento del virus variaron del 75% a los 10 días de edad al 100% a los 42 días de edad. En las aves infectadas después del nacimiento, los resultados negativos de la detección del virus J de leucosis aviar mediante la prueba de PCR y el aislamiento del virus mostraron una concordancia del 66.6% al 100% entre los 10 y 42 días. Para el aislamiento del virus se requiere de fibroblastos de embriones de pollo de líneas genéticas específicas y el proceso toma de 7 a 9 días. Adicionalmente, para la toma aséptica de sangre y tejidos, se requiere de instrumentos de necropsia estériles y de jeringas y agujas para cada ave. Por el contrario, para la detección del virus J de leucosis aviar mediante la prueba de PCR a partir de la pulpa de la pluma solo se requiere de tubos para microcentrífuga y de guantes estériles. El empleo de la prueba de PCR para la detección del virus J de leucosis aviar en los folículos de la pluma es conveniente cuando la infección por este virus debe ser diagnosticada rápida e inequívocamente sin sacrificar a las aves y en aquellas situaciones cuando los reactivos indispensables o los substratos adecuados para la propagación oportuna del virus en cultivo celular no se encuentran disponibles.

Key words: avian leukosis virus, ALV-J, diagnosis, PCR

Abbreviations: AC-ELISA = antigen-capture enzyme-linked immunosorbent assay; ALV = avian leukosis virus; ALV-J = avian leukosis virus subgroup J; GSA = group-specific antigen; MDV = Marek's disease virus; MGIF = Molecular Genetics Instrumentation Facility; PCR = polymerase chain reaction; REV = reticuloendotheliosis virus; $TCIU_{50}$ = mean tissue culture infectious units

Subgroup J avian leukosis virus (ALV-J) is an oncogenic avian retrovirus first described in 1991 (13). ALV-J infects meat-type chickens naturally, and it became widely distributed in the 1990s (6). Clinical infection with ALV-J causes economic losses by inducing an array of tumors and high mortality, primarily in mature chickens (15). Diagnosis of infection with avian leukosis viruses (ALVs) for eradication from breeding stock has been accomplished by a variety of methods (22), including virus isolation, complement fixation, and antigen-capture enzyme-linked immunosorbent assays (AC-ELISA) (4,5,9,10,11,12,16,18,19). More recently, molecular-based diagnostic methods, including *in situ* hybridization (1) and the polymerase chain reaction (PCR) (8,17,20,21), have been developed for the detection of ALV-J nucleic acids. Virus isolation from blood components is currently the standard method of virus detection used by primary breeding operations (6). The AC-ELISA test is also commonly used on a variety of samples, including cell culture fluids, meconium, and egg albumen (4,5,23). ALVs are present in the feather pulp of infected chickens, and thus the AC-ELISA test on feather pulp extracts has been used for detecting group-specific antigen (GSA) of ALVs (9,14) and for determining the genetic susceptibility to ALV in chickens (14), but its use for eradication purposes is rather uncommon and does not allow for the specific identification of the ALV subgroup infecting the chicken host. In the present study, detection of ALV-J proviral DNA was accomplished in feather pulp of chickens infected experimentally as embryos, as newly hatched chicks, or as contact-exposed chickens. Our results demonstrated that PCR for detection of ALV-J proviral DNA can be used as an alternative method for rapid identification of ALV-J-infected flocks without the usual requirement of destroying birds or culturing viruses from blood samples or tissues. PCR for detecting ALV-J proviral DNA is proposed as an alternative diagnostic assay on a flock basis but not for eradication purposes.

MATERIALS AND METHODS

Field isolates and virus cultures. The ADOL-7501 broiler isolate of ALV-J was kindly provided by Dr. Aly Fadly (USDA/ARS, East Lansing, MI). In our laboratory, the virus was first propagated in sec-

ondary embryo fibroblasts from line 0 chickens (C/E) (2) and then in ALV_A embryo fibroblasts (C/A, E) (3). Two additional passes were made in secondary fibroblasts of line 0 chickens (C/E). Positive cultures were identified by a commercial AC-ELISA system for the detection of ALV GSA (IDEXX Laboratories, Westbrook, ME). The virus was used for inoculations in its fourth passage (ADOL-7501p4) and had a titer of $10^{3.7}$ mean tissue culture infectious units ($TCIU_{50}$)/ml. Proviral DNA of infected fibroblasts from the virus stock was used to attempt detection of reticuloendotheliosis virus (REV) and Marek's disease virus (MDV). A virus neutralization assay with ALV-J-specific antiserum was performed to rule out contamination with ALVs from other subgroups in the inoculum. PCR primers specific for ALV-J were used to detect proviral DNA from ADOL-7501p4 in genomic DNA from infected fibroblasts. The complete proviral genome of ADOL-7501 has been sequenced (G. Zavala *et al.*, unpubl.), and the sequence is available from GenBank (AY027920).

Chickens. Thirty female-line broiler breeder chicks and 60 hatching eggs were obtained from a single grandparent flock. Plasma and white blood cells from the 30 birds obtained as chicks were inoculated into duplicate cultures of line 0 (C/E) secondary chicken embryo fibroblasts. All chickens sampled were free of exogenous ALV infection.

Sample collection. Feather pulp was collected from three primary wing feathers in chickens under 3 wk of age, two primary feathers in 3-to-6-wk-old chickens, or one primary feather in chickens older than 6 wk. Briefly, the feathers were handled by the shaft at a distance of at least 1 cm from their insertion into the wing. The feathers were dislodged, and approximately 3–4 mm from the feather proximal tips was placed into the mouth of a sterile microcentrifuge tube. The lid of the tube was used to compress the pulp out of its shaft as the feather was withdrawn from the tube. This procedure allows for the collection of approximately 25–50 mg of feather pulp material. The samples were centrifuged at 14,000 rpm for 1 min and stored at −80 C until processed. The DNA extraction was performed in the same sampling tube. Whole blood samples (0.75–1.5 ml) were collected with sterile syringes and needles. The blood samples were placed into sterile tubes containing sodium heparin (Beckton Dickinson Vacutainer Systems, Franklin, NJ). Within 1 hr after collection, all blood samples were centrifuged at 1500 rpm for 3 min at 4 C. The plasma and residual white blood cells were separated and inoculated immediately into duplicate cell cultures or frozen at −80 C until processed for virus isolation.

Nucleic acid extraction, amplification, and sequencing. Total cellular DNA was extracted with a commercial DNA extraction kit (High Pure PCR Template Preparation Kit; ROCHE Biochemicals, Mannheim, Germany). The DNA was kept refrigerated at 4 C in buffer until used for PCR. Two separate sets of primers were used for the detection of ALV-J. An amplicon of the expected size (~2125 bp) was obtained with the forward primer J3#1 5′GTGCGTGGTTATTATTTCC3′ and reverse primer J3#1 5′TATTGCTGTTTCATCGTTA3′ designed in our laboratory. These primers amplified specifically sequences from the ALV-J reference strains ADOL-Hc1 (7) and HPRS-103 (13) and did not amplify sequences from ALV subgroup A (RAV-1), B (RAV-2), C (RAV-49), D (RAV-50), or E (RAV-0). The second set of primers (21) produced a 545-bp amplicon and included forward primer (JH5F) 5′GGATGAGGTGACTAAGAAAG3′ and reverse primer (JH7R) 5′CGAACCAAAGGTAACACACG3′. The first primer set (J5#1:J3#1) was used for detecting ALV-J proviral DNA in feather pulp in the experiment described herein and for comparison of results with virus isolation. The second primer set (JH5F:JH7R) was used only for validating PCR results obtained with the J5#1:J3#1 primers. An additional primer set was used for amplification of part of the DR1 region, as well as the E element (XSR region) and part of the 3′LTR, with the purpose of verifying the specificity of the assay by sequencing this region directly from PCR product from induced tumors and feather pulp. This primer set was designed on the basis of the proviral DNA sequence of the ADOL-7501 isolate used in this experiment. The full proviral genome of ADOL-7501 has been sequenced (G. Zavala *et al.*, unpubl.), and the sequence is available from GenBank (AY027920). The forward primer DU5F 5′GGGCGGGCTTCGGTTGTA3′ and reverse primer DU5R 5′TCGCTCATGCAGGTGCTCGTAGTT3′ produce a 517-bp amplicon. Primers for detection of REV and serotype 1 MDV have been described previously (17). All primers were synthesized at the Molecular Genetics Instrumentation Facility (MGIF) at the University of Georgia or at Research Genetics (Huntsville, AL). For the 2125-bp amplicon, proviral DNA was amplified by standard PCR methods. The initial cycle included denaturation for 5 min at 94 C, renaturation for 3 min at 55 C, and primer extension for 8 min at 72 C. The first cycle was followed by 35 cycles of denaturation at 94 C for 15 sec, renaturation at 55 C for 2 min, and extension at 74 C for 4 min. One final PCR cycle was performed with denaturation at 94 C for 15 sec, renaturation at 55 C for 2 min, and extension at 74 C for 10 min in a Perkin Elmer GeneAmp System 9600 thermocycler (Perkin-Elmer Applied Biosystems, Foster City, CA). Primers JH5F:JH7R were used to amplify proviral DNA by following published procedures (21). Sequencing was performed by the dideoxy chain termination method at the MGIF. Sequence analysis was accomplished with

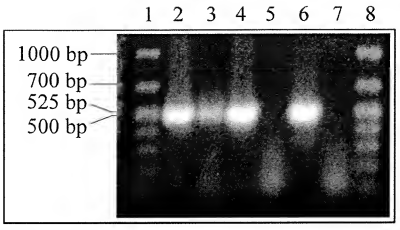

Fig. 1. 1% agarose gel displaying a 517 bp PCR product amplified from proviral DNA obtained from induced tumors and from feather pulp from four separate birds. The amplicons span part of DR1, the E element, and most of the 3′LTR. Lanes 1 and 8, DNA markers; lanes 2, 3, and 4, PCR product from proviral DNA obtained from myelocytomas induced in three separate embryo-inoculated birds and sampled at 38 days of age; lane 5, negative control (PCR mix without template); lane 6, PCR product from proviral DNA obtained from feather pulp from a 7-day-old bird inoculated at 6 days of embryonic development; lane 7, negative control (C/E chicken embryo fibroblast DNA).

the 4.03 version of the MegAlign sequence analysis software (DNASTAR, Inc., Madison, Wisconsin).

Experimental design. Thirty 1-day-old broiler breeder chicks were segregated into three experimental groups of 10 chickens each. Group 1 was a mock-inoculated control, receiving cell culture medium only as inoculum. Five chickens in group 2 were inoculated at 3 days of age with 0.2 ml of a 10^{-3} $TCIU_{50}$/ml inoculum containing the ADOL-7501p4 isolate. The remaining five chickens were mock infected and left as contacts for horizontal transmission of ALV-J. Group 3 was a replicate of group 2. All inoculations in chicks were done intraperitoneally. Fertile eggs obtained from the same grandparent flock were inoculated intrayolk at 6 days of incubation with 0.2 ml of a 10^{-2} $TCIU_{50}$/ml inoculum containing the ADOL-7501p4 isolate. The birds obtained as 1-day-old chicks ($n = 30$) and the chicks hatched in our facilities ($n = 28$) were placed in isolators for the duration of the study. The detection of ALV-J infection was attempted by virus isolation from blood and PCR on feather pulp at defined intervals. Correlation between the results of virus isolation and PCR were examined statistically by a Fisher exact test (GraphPad InStat 3.00; GraphPad Statistical Analysis Software, San Diego, CA).

RESULTS

The PCR reactions resulted in specific products of the expected sizes. In this experiment, the PCR results obtained with our primers agreed 100% with results generated with PCR primers previously described (21) on replicate feather pulp DNA samples (data not shown). For confirmation of the presence of detectable ALV-J proviral DNA in feather pulp material, a fragment of proviral DNA was amplified from induced tumors detected in the experimentally inoculated chickens and from feather pulp. This fragment of 517 bp spans part of the DR1, XSR (E element), and LTR regions of ALV-J. The amplicons obtained from three tumors induced in three separate chickens and from one feather pulp sample from a chicken inoculated with ADOL-7501p4 were of the expected size (~517 bp), with the DU5F:DU5R primers (Fig. 1). The ALV-J proviral nucleotide sequences obtained from the tumors and feather pulp were 99.8%–100.0% identical to the corresponding sequence of ADOL-7501 (GenBank AY027920) and ≥95% identical to the HPRS-103 prototype strain of ALV-J (GenBank Z46390) (data not shown).

None of the feather pulp samples from chickens inoculated as embryos rendered ALV-J-positive results by 2 days of age, a period when it was difficult to collect enough feather pulp material for a positive identification by

Table 1. Summary of results for chickens infected as embryos.

Age (days)	Group 4 (embryo inoculated)[A]	
	PCR positive[B]	Virus isolation positive[C]
2	0/10	25/25
7	27/28	26/27
28	23/23	23/23
42	16/16	16/16
49	14/14	14/14
56	8/9[D]	12/12

[A]Chickens were inoculated as embryos at 6 days of incubation.
[B]Number positive/number tested, primers J5#1 and J3#1 (~2125 bp).
[C]Number positive/number tested.
[D]Only nine plasma samples were tested at 56 days of age.

PCR (Table 1) because the feathers and feather follicles were very small. By 7 days of age, ALV-J was detected in the feather pulp of 96% of the chickens inoculated as embryos and in all embryo-inoculated chickens at 28, 42, 49, and 56 days of age. By virus isolation on samples collected between 7 and 56 days of age, ALV-J was detected in 96% or more of the chickens infected as embryos (Table 1).

For the chickens inoculated at 3 days of age, ALV-J was detected by PCR on feather pulp in 0 and 60% of the chickens at 3 and 10 days of age, respectively. The PCR test was positive for 100% of the chickens inoculated after hatch in all samples collected between 21 and 42 days of age. Virus isolation was positive for 0 and 70% of the chickens inoculated posthatch at 3 and 10 days of age, respectively. Only 10% of the contact-exposed chickens were PCR positive by 10 days of age, but by 42 days of age, 33.33% of them were PCR positive. By virus isolation, all contact-exposed chickens remained negative until 10 days of age. Between 21 and 42 days of age, the percentages of viremic chickens ranged between 11% and 40% in the contact-exposed groups, as assessed by virus isolation from plasma and white blood cells (Table 2). The results obtained by PCR on feather pulp samples were not statistically different from the results obtained by virus isolation for the chickens inoculated as embryos (Table 1). In the contact-exposed chickens, PCR and virus isolation results were not statistically different at 10, 28, and 42 days of age (Table 2). Detection by PCR and virus isolation in contact-exposed chickens was statistically different only at 21 days of age, when virus isolation was more sensitive.

DISCUSSION

ALV-J was specifically detected in feather pulp from experimentally infected chickens, as demonstrated by high nucleotide sequence identity of a 517-bp amplicon obtained from feather pulp and tumor proviral DNA of four chickens inoculated experimentally with the ADOL-7501p4 strain of ALV-J. The four ADOL-7501p4 nucleotide sequences from tumors and feather pulp showed identities of 99.8%–100% between them and also with ADOL-7501 (GenBank AY027920). The specificity of the assay was confirmed by repeated unsuccessful detection in feather pulp from mock-infected chickens or from genomic DNA obtained from uninfected chick embryos (data not shown). In addition, the PCR results ob-

Table 2. Summary of results for birds infected posthatch.

Age (days)	PCR positive[A]			Virus isolation positive[B]		
	Controls	Inoculated[C]	Contact exposed	Controls	Inoculated[C]	Contact exposed
3	0/10	0/10	0/10	0/10	0/10	0/10
10	0/10	6/10	1/10	0/10	7/10	0/10
21	0/9	9/10	0/10	0/9	10/10	4/10
28	0/9	10/10	0/9	0/9	10/10	1/9
42	0/9	10/10	3/9	0/9	10/10	3/9

[A]Number positive/number tested, primers J5#1 and J3#1 (~2125 bp).
[B]Number positive/number tested.
[C]Birds were inoculated with ALV-J (ADOL-7501p4) at 3 days of age.

tained with our primers were validated by a 100% correlation of positive and negative results with PCR results obtained with previously published PCR oligonucleotide primers (21) (data not shown). In this experiment, the sensitivity of the PCR assay as compared with virus isolation can be assessed only by comparing the number of infected chickens detected by either method and under the conditions of the experiment. No attempt was made to determine the sensitivity of the assay in terms of the ability to detect limiting target DNA copy numbers because the primary objective was to develop a molecular-based diagnostic technique for detecting ALV-J in groups of chickens rather than in individual birds. PCR detection of ALV-J in feather pulp is proposed as an alternative method that can be used to detect ALV-J proviral DNA in feather pulp that can be easily sampled and transported to the laboratory without the need to use sterile syringes and blood collection tubes for virus isolation and without having to kill birds to obtain tissue samples for PCR. The PCR results presented herein were generated with primers that were originally designed to amplify a rather long DNA fragment (~2125 bp) for sequencing purposes. In our laboratory, our primers have successfully amplified proviral DNA from ALV-J isolates that could not be detected by previously published primers and PCR conditions (20). The proviral DNA of such isolates has been confirmed in our laboratory to exhibit high identity with the ADOL-7501, ADOL-Hc1, and HPRS-103 isolates of ALV-J (G. Zavala *et al.*, unpubl.). In addition, our primers have been used successfully to amplify diagnostically and in research conditions proviral DNA of the ADOL-7501 isolate and of ALV-J isolates from multiple generations and strains of meat-type chickens produced by various primary breeders (data not shown). Therefore, we chose to use our primers instead of previously published primers for the present research. However, we used previously published primers and sequencing to validate the specificity of our results. One important objective of this research was to demonstrate the applicability of PCR on feather pulp DNA extracts for diagnosing ALV-J infection rather than to propose our very own primers as the sole primer set possibility for this objective. Other primer sets could potentially be used with equal or better success, especially because our primers generate a rather large amplicon.

ALV-J was detected as early as 7 days of age in most of the chickens infected as embryos (96%). Earlier detection was limited, possibly by the lack of sufficient feather pulp material in the shafts of the primary feathers. In our hands, sampling one or two feathers rendered approximately 25–50 μg of feather pulp material between the ages of 1 and 7 wk (data not shown). Before 1 wk of age, the feathers were very small, thus limiting substantially the amount of feather pulp material available for sampling and DNA extraction. In addition, sufficient concentration of proviral DNA in the feather pulp may require an extended period of time after infection for successful detection, but this remains to be investigated. Positive detection of infected chickens by PCR on feather pulp DNA was partially compromised in birds over 49 days of age, possibly because of the significant reduction of fresh feather pulp material that occurs naturally in primary feathers at around 7–8 wk of age. At this age, most of the primary feathers in the chickens in this experiment did not contain a significant amount of pulp suitable for sampling and DNA extraction. On the basis of our data, the optimal age range for detection of ALV-J in feather pulp from primary feathers in young chickens appears to be between 1 and 7 wk of age. PCR-based detection of ALV-J in feather pulp at an age later than 7 wk could be attempted with feathers from other anatomic sites that exhibit a more active feather growth than the primary feathers to warrant harvesting sufficient fresh feather pulp material, but this remains to be investigated. ALV-J was detected by PCR 7 days postinoculation in 60% of the chickens infected at 3 days of age, suggesting that this technique could be used to detect ALV-J soon after infection. PCR detection of ALV-J proviral DNA in the feather pulp of contact-infected chickens may have been less successful because the viral concentration may have been lower in the feather follicles and also possibly because of the fact that infection occurred much later than in the chickens infected as embryos. In the natural infection, there may be a low provirus concentration in the feather pulp, thus making it more difficult to detect ALV-J proviral DNA by PCR, as was suggested by the results obtained from the contact-exposed chickens. Thus, a potential

disadvantage of the use of PCR for detecting ALV-J in feather pulp is that the assay may not be as sensitive as virus isolation in the natural infection and under field conditions. Another disadvantage is the fact that the assay detects ALV-J-infected chickens specifically, but it would not detect avian leukosis viruses of other subgroups that could potentially infect the same chickens. In addition, detection of ALV-J by PCR in feather pulp requires the presence of a sufficient amount of fresh pulp material in the primary feathers for DNA extraction, which in this experiment was optimal only between 1 and 7 wk of age, a rather narrow age window. An additional drawback of PCR as the sole form of ALV identification is that ALVs from other subgroups would not be detected should they be present in the feather pulp samples. Advantages of ALV-J detection in feather pulp by PCR include ease and speed of sample collection, and there is no need for syringes, needles, and blood collection tubes with anticoagulant. ALV-J can be identified specifically in feather pulp samples within hours as opposed to days or weeks required for virus isolation. Discrimination of replication-competent endogenous ALVs requires the use of chicken cells that are permissive to exogenous viruses only, and such cells are not always readily available. In addition, the samples may be stored frozen without any further processing before performing the actual PCR assay. Finally, there is no need to kill the chickens to be tested and virus isolation may be obviated. Under the conditions of this experiment, ALV-J detection in feather pulp by PCR offered an acceptable correlation with virus isolation in young chickens, particularly at an age when feathers were growing actively and contained abundant feather pulp material. However, the assay is hereby recommended as a diagnostic tool but not for eradication purposes. Detection of ALV-J proviral DNA in feather pulp can be used in research situations or where virus isolation is difficult to accomplish.

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